Synthesis of Tritium-Labeled Hydroxytyrosol, a Phenolic Compound Found in Olive Oil

Kellie L. Tuck,* Hai-Wei Tan, and Peter J. Hayball*

Centre for Pharmaceutical Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, 5000, Australia

(3,4-Dihydroxyphenyl)ethanol, commonly known as hydroxytyrosol (**1**), is the major phenolic antioxidant compound in olive oil, and it contributes to the beneficial properties of olive oil. Bioavailability and metabolism studies of this compound are extremely limited, in part, related to unavailability of radiolabeled compound. Studies with radiolabeled compounds enable use of sensitive radiometric analytical methods as well as aiding elucidation of metabolic and elimination pathways. In the present study a route for the formation of hydroxytyrosol (**1**), by reduction of the corresponding acid **2** with tetrabutylammonium boronate, was found. Methods for the incorporation of a tritium label in **1** were investigated and successfully accomplished. Tritiated hydroxytyrosol (**1t**) was synthesized with a specific activity of 66 Ci/mol. The stability of unlabeled and labeled hydroxytyrosol was also investigated.

Keywords: *Hydroxytyrosol; olive oil; deuteriation; tritiation; tetrabutylammonium boronate*

INTRODUCTION

(3,4-Dihydroxyphenyl)ethanol (**1**), also known as hydroxytyrosol, is one of the major phenolic compounds present in olive oil (Fedeli, 1979). Epidemiological studies have linked the high dietary intake of natural antioxidants, found in olive oil with a lower incidence of coronary heart disease (Keys, 1970; Keys, 1995) and certain cancers, namely prostate and colon cancers (Martin-Moreno et al, 1994; Lipworth et al, 1997). Hydroxytyrosol (**1**), a phenol, is one of the major antioxidants present in olive oil (Montedoro, 1972). However, despite the reported importance of the biological properties of phenols and polyphenols, published data on the absorption and disposition of **1** are scarce (Visioli et al, 1998; Visioli et al, 2000). This lack of data is mainly because **1**, which is easily oxidized, is not available commercially and because a sensitive method for the determination of **1** in biological matrixes is limited. A GC-MS quantitative analysis procedure has been used to measure the concentration of **1** in rat plasma after it was orally administered (Bai et al, 1998). However, GC-MS was not available in our laboratory and we investigated an alternative technique to analysis by GC-MS. One method of increasing the sensitivity of detection is with the use of radiolabeled compounds. Tritium-labeled compounds often have high specific activities and can be introduced postsynthetically, for example with the use of isotopic exchange reactions.

Hydroxytyrosol has been previously synthesized from (3,4-dihydroxyphenyl)acetic acid (**2**) by two routes: direct reduction with lithium aluminum hydride (Baraldi et al, 1983) and reduction with (trimethylsilyl)diazomethane and sodium borohydride (Bai et al, 1998). Also, the methyl ester of (3,4-dihydroxyphenyl)acetic acid can be reduced with lithium aluminum hydride to directly give **1** (Verhe et al, 1992). However, all of these reactions, except one (Bai et al, 1998), give poor yields of **1** and none describe the introduction of a tritium label.

In an effort to investigate the biological properties of **1**, we examined two different syntheses for its facile formation from **2**. Specifically, we wish to examine the bioavailability and the pharmacokinetic properties of **1** after oral and parenteral administration. We also plan to investigate the metabolic profile of this pivotal compound found in olive oil. Two routes for incorporation of a tritium label in **1** were explored, and we would like to disclose a successful procedure for the synthesis of tritiated hydroxytyrosol (**1t**).

MATERIALS AND METHODS

Reagents. (3,4-Dihydroxyphenyl)acetic acid (**2**), $NaBH_4$, $NaBD_4$ (98%), deuterium oxide (99.9%), and $LiAlH_4$, 1.0 M solution in THF, were obtained from Aldrich (Sydney, Australia). THF was freshly distilled from sodium/benzophenone prior to use. All other chemicals and solvents were of analytical grade or higher and used without purification. Organic solutions were dried over $MgSO_4$, unless otherwise stated. Chromatography was performed with silica gel (230−400 mesh). TLC was performed with Merck DC Alufolien Kieselgel 60 F_{254}, which was visualized either with UV light or by immersion in acidic ammonium molybdate solution.

Apparatus. NMR spectra were measured with a Varian spectrometer with an operating frequency of 200 MHz; acetone-d_6 or water-d_2 was used as the solvent. HPLC analysis and sample collection were performed on a Hewlett Packard 1100 Series system consisting of a 1100 series isocratic pump, 1100 series autosampler, and 1100 series variable wavelength detector and with a semipreparative SymmetryPrep C_{18} 7 μm (7.8 mm × 300 mm) column or an analytical DuPont Zorbax phenyl (4.6 mm × 25 cm) column. The compounds were detected at 281 nm. Specific activities were measured by preparing solutions of hydroxytyrosol (**1**) of known concentration, in scintillant, and radiometrically counting the aliquots using a PACKARD Tri-Carb 2000CA liquid scintillation

* Corresponding authors: K.L.T., Ph 61 8 83022301, fax 61 8 83022389, e-mail Kellie.Tuck@unisa.edu.au; P.J.H., Ph 61 8 83021646, fax 61 8 83022389, e-mail Peter.Hayball@unisa.edu.au.

10.1021/jf000468l CCC: $19.00
Published on Web 08/09/2000

counter. HPLC radiometric analysis was performed with a Radiomatic 150TR-flow scintillation analyzer (scintillant flow 2.5 mL/min and HPLC flow 1 mL/min).

Preparation of Hydroxytyrosol with $LiAlH_4$. (3,4-Dihydroxyphenyl)acetic acid (**2**) (0.5 g, 3.0 mmols) dissolved in THF (2.0 mL) was added to an ice-cooled solution of 1.0 M $LiAlH_4$ in THF (4.5 mL, 4.5 mmol). After the addition was complete, the suspension was heated under reflux for 3 h and cooled in an ice bath, and excess $LiAlH_4$ was destroyed by the careful addition of H_2O (10 mL) and 1.0 M HCl (10 mL). The mixture was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were dried and concentrated in vacuo. Careful flash chromatography (ethyl acetate/hexanes, 50/50 v/v) gave **1** as a colorless oil (45%). δ_H: 2.70 (2H, t, J = 6.8 Hz, H1′), 3.75 (2H, t, J = 6.8 Hz, H2′), 6.70 (1H, dm, J = 8.0 and 2.0 Hz, H6), 6.80 (1H, d, J = 2.0 Hz, H2), 6.84 (1H, d, J = 8.0 Hz, H5).

Preparation of Hydroxytyrosol (1) with Tetrabutylammonium Boronate. *Preparation of Tetrabutylammonium Boronate.* Tetrabutylammonium hydrogen sulfate (0.34 g, 1.0 mmols) dissolved in H_2O (0.2 mL) was added to 5.0 M NaOH (0.25 mL). To the mixture, cooled in a slurry of ice, was added $NaBH_4$ (0.04 g, 1.1 mmol) dissolved in H_2O (0.1 mL). After stirring of the reaction mixture for 5 min, it was extracted with CH_2Cl_2 (3 × 5 mL). The combined organic extracts were dried and concentrated in vacuo to approximately $^1/_3$ of the original volume. The solution was then used directly in the next step.

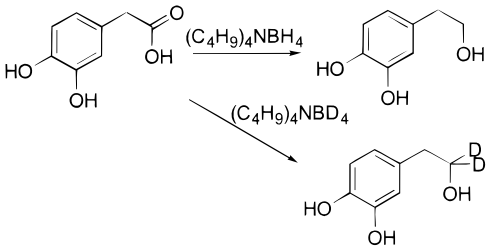
*Preparation of **1** with Tetrabutylammonium Boronate.* To a round-bottom flask containing the above solution of tetrabutylammonium boronate (1.0 mmols) was added (3,4-dihydroxyphenyl)acetic acid (**2**) (0.84 g, 0.5 mmol) dissolved in THF (0.2 mL). The flask was cooled in an ice bath, and MeI (0.15 mL, 2.0 mmol) was carefully added, so to avoid the excess evolution of methane. After being stirred for 2 h at room temperature, the excess hydride was destroyed by the careful addition of ethanol (10 mL) and 1.0 M HCl (10 mL) to the ice-cooled solution. The solution was extracted with ethyl acetate (3 × 30 mL), and the combined organic extracts were dried, and concentrated in vacuo. Column chromatography (ethyl acetate/hexanes, 50/50 v/v) gave the product as a light yellow oil (99%). δ_H: 2.70 (2H, t, J = 6.8 Hz, H1′), 3.75 (2H, t, J = 6.8 Hz, H2′), 6.70 (1H, dm, J = 8.0 and 2.0 Hz, H6), 6.80 (1H, d, J = 2.0 Hz, H2), 6.84 (1H, d, J = 8.0 Hz, H5).

Preparation of Deuterated Hydroxytyrosol (1a) with Deuterated Tetrabutylammonium Boronate. The above procedure was repeated with tetrabutylammonium hydrogen sulfate (3.4 g, 10.0 mmol), D_2O (2.0 mL), 5.0 M NaOH (2.5 mL), $NaBD_4$ (0.4 g, 11.0 mmol), and D_2O (2.0 mL). A $^1/_{10}$ amount of this mixture was reacted with **2** (0.84 g, 0.5 mmols) dissolved in THF (0.2 mL). After workup and concentration the deuterated compound **1a** was obtained as a yellow oil in a 56% yield. δ_H: 2.70 (2H, s, H1′), 6.70 (1H, dm, J = 8.0 and 2.0 Hz, H6), 6.80 (1H, d, J = 2.0 Hz, H2), 6.84 (1H, d, J = 8.0 Hz, H5).

Deuteriation of 1 with Amberlyst 15. *(i) Reaction with D_2O.* **1** (24.5 mg), Amberlyst 15 (25.2 mg), D_2O (0.2 mL), and a magnetic stirrer (7 mm) were introduced into a Hewlett Packard HPLC vial (2 mL capacity). The flask was evacuated with N_2, the top was screwed on, and the flask was placed into an oil bath. The reaction was heated at 90 °C, with continuous stirring, for 24 h. On completion the tube was cooled, the solution removed, and H_2O (3 × 0.2 mL) added to the flask to rinse the resin; this solution was removed and added to the initial solution. Freeze-drying of the solution gave deuterated hydroxytyrosol (**1d**) in a recovery yield of 98%. Analysis by ^{1}H NMR spectroscopy showed complete disappearance of the aromatic protons. δ_H: 2.70 (2H, t, J = 6.8 Hz, H1′), 3.75 (2H, t, J = 6.8 Hz, H2′).

(ii) Reaction with 50% D_2O/H_2O. The above procedure was repeated with **1** (10.9 mg), Amberlyst 15 (12.0 mg), D_2O (0.1 mL), and H_2O (0.1 mL). The substrate **1d** was obtained in a 66% recovery yield. δ_H: 2.70 (2H, t, J = 6.8 Hz, H1′), 3.75 (2H, t, J = 6.8 Hz, H2′), 6.70 (0.67H, dm, J = 8.0 and 2.0 Hz, H6), 6.80 (0.67H, d, J = 2.0 Hz, H2), 6.84 (0.67H, d, J = 8.0 Hz, H5).

Scheme 1



1a

Tritiation of 1 with Amberlyst 15. 1 (46.4 mg), Amberlyst 15 (51.1 mg), T_2O (5 mCi/mL, 0.2 mL, 1 Ci), and a magnetic stirrer (7 mm) were introduced into a Hewlett-Packard HPLC vial (2 mL capacity). The flask was evacuated with N_2, the top was screwed on, and the flask was placed into an oil bath. The reaction was heated at 90 °C, with continuous stirring, for 24 h. On completion the tube was cooled, the solution removed, and H_2O (1 mL) added to the flask to rinse the resin; this solution was removed and added to the initial solution. This procedure was repeated three times, to ensure complete removal of the substrate. Freeze-drying of the solution gave tritiated hydroxytyrosol (**1t**) in a recovery yield of 97%. The residue was dissolved in H_2O (2 mL) and purified by preparative HPLC (95 v/v H_2O (containing 2/5 v/v acetic acid/MeOH, 2 mL/min). The eluted substrate in mobile phase (85 mL) was extracted with ethyl acetate (4 × 80 mL). The combined organic phases were dried (Na_2SO_4), and solvent was removed in vacuo. The specific activity of **1t** was found to be 66 Ci/mol.

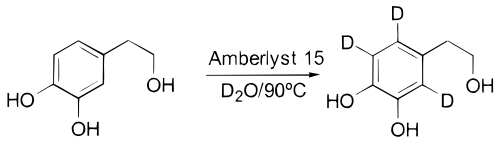
Stability of 1 at pH 8.85. 1 (1.8 mg) was added to colonic lavage buffer (5 mL) (Davies et al, 1980). The resulting solution had a pH of 8.85. Aliquots (100 μL) were added to ependorf tubes and incubated at 37 °C. At time intervals of 0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, and 120 min the ependorf solutions were diluted with mobile phase (99.5 v/v H_2O (containing 0.2/0.5 v/v acetic acid)/MeOH, 1 mL/min). These solutions were further diluted (10×) with mobile phase and analyzed by HPLC. The results are summarized in Figure 3.

RESULTS AND DISCUSSION

A sample of hydroxytyrosol (**1**) was obtained by reduction of (3,4-dihydroxyphenyl)acetic acid (**2**) with lithium aluminum hydride; spectral data were comparable to that which had previously been published (Montedoro et al, 1993). However, optimization of this reaction gave **1** in only a 45% yield after purification by chromatography. It must be noted that care needs to be taken during the chromatography as **1** is not stable on silica. Consequently chromatographic purification must be performed quickly so that the yield of **1** is optimized.

In an effort to improve the yield of **1** from its corresponding acid **2** an alternative reduction reaction was investigated. Tetrabutylammonium boronate is a reducing reagent of esters (Brändström et al, 1972) and gives the corresponding alcohols in yields of 80−98%. Tetrabutylammonium boronate, formed by reaction of tetrabutylammonium hydrogen sulfate with sodium borohydride, does reduce **2** to give **1** in a 99% yield (Scheme 1); spectral data correspond to that of **1** synthesized previously. It was recognized at this stage, with the use of deuterated or tritiated sodium borohydride, that it was possible to incorporate a label at the 2′ position in **1**. Indeed, reaction of 3,4-dihydroxyphenylacetic acid with tetrabutylammonium boronate, formed by reaction of tetrabutylammonium hydrogen sulfate with sodium borodeuteride, gave the corresponding deuterated hydroxytyrosol (**1a**) in a 56% yield. The

Scheme 2



1 **1d**

^{1}H NMR spectrum of **1a** showed disappearance of the triplet at 3.75, and the triplet previously at 2.70 was now a singlet. Hence, a deuterium had been successfully incorporated at the 2′ position.

Consequently, there now exists a procedure for the introduction of a deuterium at the 2′ position of **1** and this procedure can be applied to the tritiation of **1**. Unfortunately, sodium borotritide is very expensive, and hence, an alternative cheaper route for the tritiation of **1** was investigated. Nevertheless, an alternative, efficient route for the synthesis of **1** from its corresponding acid **2** has been found.

There have been recent reports in the literature of the use of ion-exchange resins, in the acid form, to deuterate and tritiate a large number of molecules (Brewer et al, 1994). We have recently modified this technique and have applied it, with the use of the polymer supported acid catalyst Amberlyst 15, to deuterate a large number of phenolic compounds (Tuck et al, 2000). We now wish to report the synthesis of tritiated hydroxytyrosol (**1t**) using Amberlyst 15.

We initially deuterated rather than tritiated **1** due to the wide availability, high isotopic purity, and affordability of deuterated water and the fact that the reaction could be monitored by ^{1}H NMR spectroscopy. Reaction of **1** with Amberlyst 15 and deuterium oxide, after heating for 24 h, resulted in full exchange of the aromatic protons at the 2, 5, and 6 positions of the aromatic ring (Scheme 2). However, when the reaction was stopped after 15 h the incorporation was 100% at C6, 70% at C2, and 15% at C5. The difference in the rate of deuteriation is due to the relationship of the proton to the hydroxyl groups present in the substrate. In these compounds the *ortho* and *para* positions are mesomerically activated and the protons in these positions will exchange preferentially; this is in accord with an electrophilic substitution type mechanism. Reaction of **1** with a solution of H_2O/D_2O (1:1), 24 h of heating, gave a 33% incorporation of a deuterium at C6, C2, and C5.

It was discovered that up to 50 mg of substrate could be effectively deuterated with only 0.2 mL of deuterium oxide and by optimization of the extraction procedure recovery yields as high as 98% were obtained.

With optimization of the deuteriation conditions, the reaction was attempted with tritiated water. Reaction of **1** with tritiated water (5 mCi/mL) gave tritiated **1t** in a recovery yield of 97%. Radiolabeled **1t** was purified by preparative HPLC; after extraction and concentration the specific activity was found to be 66 Ci/mol. Figures 1 and 2 show the radiometric chromatograms of **1t** before and after purification; the peak at 3.5 is due to residual tritiated water, and the peak at 6.5 is due to **1t**. Figure 2 shows the radiometric chromatogram of **1t** after its purification by preparative HPLC.

The stability of unlabeled **1** was investigated in a number of solutions. An aqueous solution of **1** (50 μg/mL) showed no degradation products by HPLC, even after storage at 4 °C for 5 months. **1** was also stable in this aqueous solution when heated at 37 °C for 2 h. The stability of **1** (50 μg /mL) was also examined in an acidic solution (pH 3.1); after several months storage at 4 °C no degradation was seen by HPLC. However, in a basic solution (pH 8.85), at 37 °C, **1** (36 μg/mL) quickly degrades ($t_{1/2}$ = 92 min) to an unknown product. The results are represented in Figure 3.

The stability of the tritium label in **1t** was also investigated. Tritiated **1t** was stable in aqueous and acidic (pH 3.1) solutions, at 4 °C, for a number of weeks. No exchange of the label was observed by radiometric HPLC. Also, no change in the specific activity of **1t** was

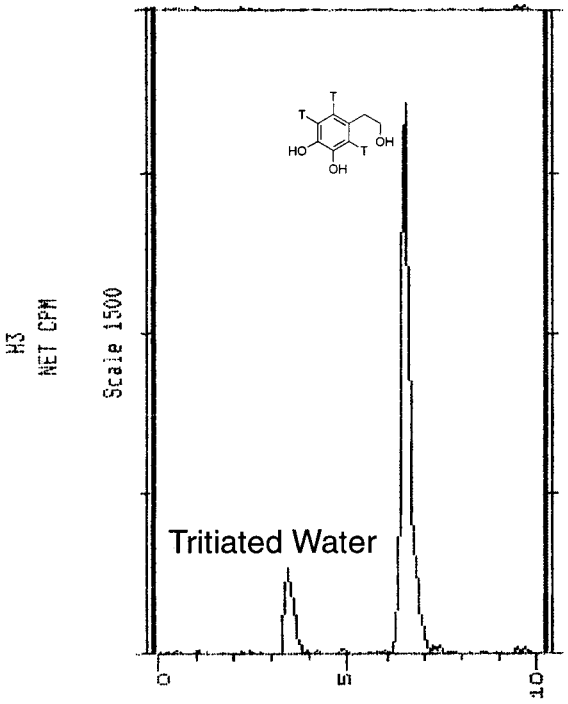


Figure 1. Radiometric chromatogram of **1t** before purification by preparative HPLC.

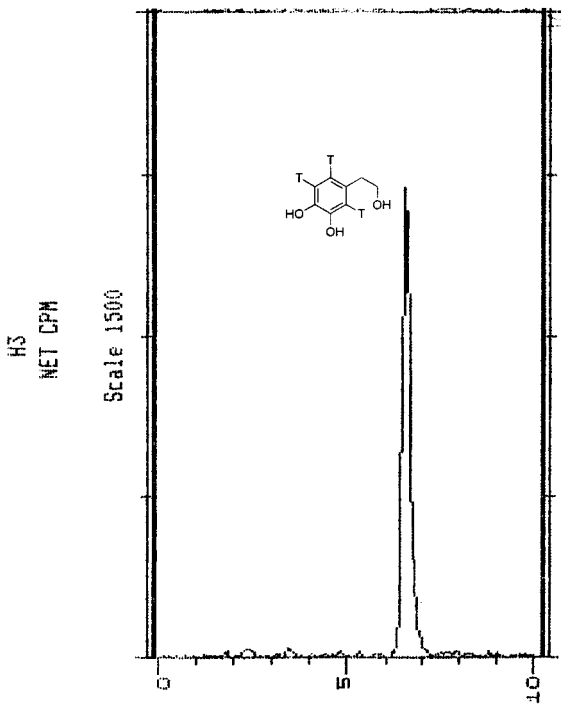


Figure 2. Radiometric chromatogram of **1t** after purification by preparative HPLC.

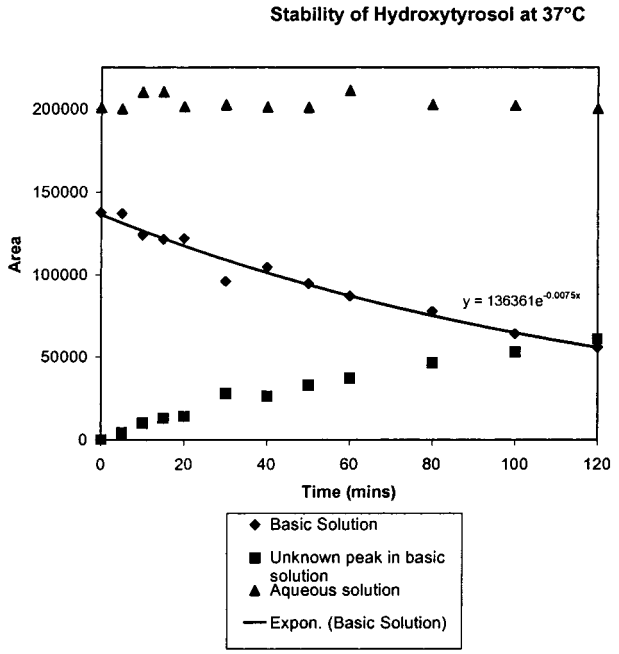


Figure 3. Degradation of **1** in a basic solution (◆) (pH 8.85) at 37 °C and the nondegradation of **1** in an aqueous solution (▲) at 37 °C.

observed after storage, in either its neat form or as an aqueous solution, at −20 °C for 2 months.

In conclusion, a facile route for the synthesis of **1** from its corresponding acid **2** with the use tetrabutylammonium boronate has been found. Tritiated **1t**, with a high specific activity, 66 Ci/mol, can be synthesized from unlabeled **1** with the use of Amberlyst 15 as a polymer supported acid catalyst. Elucidation of the absorption, metabolism, and disposition of hydroxytyrosol should now be possible. Numerous studies (Manna et al, 1997; Visioli et al, 1998) have shown that phenolic compounds possess strong radical scavenging activity and appear to be at least as, if not more, effective compared to other important dietary antioxidants such as vitamin C and α-tocopherol.

LITERATURE CITED

Baraldi, P. G.; Simoni, D.; Manfredine, S.; Menziani, E. Preparation of 3,4-dihydroxy-1-benzeneethanol: a reinvestigation. *Liebigs Ann. Chem*. **1983**, *83*, 684−686.

Bai, C.; Yan, X.; Takenake, M.; Sekiya, K.; Nagata, T. Determination of synthetic hydroxytyrosol in rat plasma by GC-MS. *J. Agric. Food Chem*. **1998**, *46*, 3998−4001.

Brändström, A.; Junggren, U.; Lamm B. An improved method for the preparation of solutions of diborane. *Tetrahedron Lett*. **1972**, *31*, 3173−3176.

Brewer, J. R.; Jones, J. R.; Lawrie, K. W. M.; Saunders: D.; Simmonds, A. Tritiation of organic compounds by polymer-supported acid catalysts. *J. Labelled Compd. Radiopharm*. **1994**, *34*, 391−400.

Davis, G. R.; Santa Ana, C. A.; Morawski, S. G.; Fordtran, J. S. Development of a lavage solution associated with minimal water and electrolyte absorption or secretion. *Gastroenterology* **1980**, *78*, 991−5.

Fedeli, E. Lipids of olives. *Prog. Chem. Fats other Lipids* **1979**, *15*, 57−74.

Keys, A. Coronary heart disease in seven countries. *Circulation* **1970**, *41*, 1−211.

Keys, A. Mediterranean diet and public health-personal reflections. *Am. J. Clin. Nutr*. **1995**, *61*, 1321S-1323S.

Lipworth, L.; Martinex, M. E.; Angell, J.; Hsien, C. C.; Trichopouls, D. Olive oil and human cancer: an assessment of the evidence. *Prev. Med*. **1997**, *26*, 181−190.

Martin-Moreno, J. M.; Willett, W. C.; Gorgojo L.; Banegas, J. R.; Rodriguez-Artalejo, F.; Fernandex-Rodriguez, J. C.; Maisonneuve, P.; Boyle, P. Dietary fat, olive oil intake and breast cancer risk. *Int. J. Cancer* **1994**, *58*, 774−780.

Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. The protective effect of olive oil polyphenol (3,4-dihydroxyphenyl)ethanol counteracts reactive oxygen metabolite-induced cytotoxicity in Caco-2 cells. *J. Nutr*. **1997**, *127*, 286−292.

Montedoro, G. Phenolic substances present in virgin olive oil. Note I. Identification of phenolic acids and their antioxidant power. *Sci. Technol. Aliment*. **1972**, *2*, 177−186.

Montedoro, G. Simple and hydrolyzable compounds in virgin olive oil 3. Spectroscopic characterizations of the secoiridoid derivatives. *J. Agric. Food Chem*. **1993,** *41*, 2228−2234.

Tuck, K. L.; Tan, H.; Hayball, P. J. A simple procedure for the deuteriation of phenols. *J. Labelled Compd. Radiopharm*. **2000**, *43*, 817−23.

Verhe, R.; Papadopoulos, G.; Boskou, D. Preparation of hydroxytyrosol. *Bull. Liason−Groupe Polyphenols* **1992**, *15*, 237−244.

Visioli, F.; Galli, C. The effect of minor constituents of olive oil on cardiovascular disease-new findings. *Nutr. Rev*. **1998**, *56*, 142−147.

Visioli, F.; Galli, C.; Bornet, F.; Mattei, A.; Patelli, R; Galli, G; Curoso, D. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett*. **2000**, *468*, 159−160.

Received for review April 12, 2000. Revised manuscript received June 6, 2000. Accepted June 7, 2000.

JF000468L